

Effects of Ethylene Glycol Monomethyl (EGME) and Monoethyl (EGEE) Ethers on the Immuno-competence of Allogeneic and Syngeneic Mice Bearing L1210 Mouse Leukemia

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The effect of ethylene glycol monomethyl ether (EGME) and ethylene glycol monoethyl ether (EGEE) on cell-mediated immunity was evaluated by an allograft rejection assay. Allogeneic B6C3F₁ (C57BL/6 × C3H) mice were given oral doses of 600, 1200, or 2400 mg/kg/administration of EGEE or 300, 600, 1200 mg/kg/administration of EGME on days -12 through -8 or cyclophosphamide (Cy) at 180 mg/kg by the IP route on day -1. Untreated controls were given oral doses of water on days -12 through -8 and -5 through -1. On day 0, the mice were challenged with 1×10^2 , 3×10^3 , and 1×10^5 or 3×10^6 L1210 cells by the IP route. Syngeneic CD2F₁ (Balb/c × DBA/2) mice were challenged with 1×10^5 L1210 cells on day 0 and were treated on days 1 to 5 and 8 to 12 with the same dosages of EGME and EGEE used for the B6C3F₁ mice. Water-treated syngeneic mice died with a median survival time (MST) of 8.0 days. There was no effect on the MST of syngeneic mice treated with either EGME or EGEE, indicating no direct antitumor effect of the compounds. All allogeneic mice receiving either water or Cy and challenged with 3×10^6 tumor cells, died with ascites. However, when mice were treated with EGME or EGEE and challenged with 3×10^6 tumor cells, no more than one animal per group died. This would indicate that there was a prophylactic action of the compounds or that the immune system was stimulated. Blood smears of allogeneic mice were made for differential counts the last day of drug dosing, the day of death where possible, and on survivors at day 43 post-tumor implantation. Differential counts showed evidence of monocytosis, considered to be indicative of monocytic leukemia, in those mice not surviving until the day of sacrifice. All surviving allogeneic mice were sacrificed and autopsied on day 43. Of those receiving EGEE, 7% had cholecystitis while 58% of those receiving EGME had cholecystitis.

Introduction

In recent years, routine methods, based primarily on animal death and/or histopathological changes, have evolved to assess the toxicity and/or carcinogenicity of environmental pollutants. These approaches have aided in the identification of certain highly toxic environmental contaminants with acute effects (1). The development of assays and/or batteries of techniques to determine the effects of these agents on more subtle cell functions has not evolved as rapidly, and indeed in

many instances could proceed only after assays were developed to determine the mechanism(s) of cellular functions, differentiation, and interactions.

Attempts to apply assays of immune function to assess the effects of selected environmental pollutants on this key biological system have demonstrated the immunosuppressive effects of heavy metals (2), polychlorinated biphenyls (3), pesticides (4), fossil fuel combustion products, polycyclic aromatic hydrocarbons, to mention a few, and in selected studies the mechanism(s) of these effects have been further clarified (1). These types of studies coupled with the fact that accurate and sensitive assays of immune function have been (and continue to be) developed clearly indicate that immunotoxicology can and should be further developed and applied.

A variety of toxicological effects have been observed with ethylene glycol monomethyl ether (EGME) and ethylene glycol monoethyl ether (EGEE) including testicular atrophy (5), adverse reproductive effects

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(6,7), leukopenia (8), and thymic atrophy (9). Although no mechanistic explanations have been suggested as a common thread to explain these diverse observations, a simplistic hypothesis is that there may be direct cytotoxic effect on some rapidly growing tissues. Considering the contention of Trosko et al. (10) that interruption of intercellular communication may play a similar role in teratogenesis as well as tumor promotion and that various glycol ethers have been demonstrated to block gap junction-mediated intercellular communication (11), the glycol ethers were judged to be likely candidates for tumor promoting effects. In addition the observations of thymic atrophy in mice and rats also suggested the possibility of increased risk of tumor development through a decrease in thymus dependent cellular immunity. We chose to assess the effects of these chemicals on the immune system using an allogeneic tumor challenge model (12). In this model, mice which are allogeneic in relation to the leukemic cell tumor used will not die when challenged unless they have been immunosuppressed, while in syngeneic mice the tumor will grow and kill the animals unless there is a direct cytotoxic effect as a result of chemical treatment on the tumor cells.

Materials and Methods

The test compounds, ethylene glycol monoethyl ether (CAS No. 110-80-5) and ethylene glycol monomethyl ether (CAS No. 109-86-4) were purchased from Aldrich (lot Nos. 0914EH and 3623ME, respectively). Commercially available cyclophosphamide (Mead Johnson) was used as a positive control, and distilled water was used as the negative control compound and for the dilution vehicle.

The mouse lymphoid leukemia L1210 was utilized in this study. Obtained originally from the National Cancer Institute (NCI), this ascitic tumor is propagated serially by intraperitoneal (IP) implantation in DBA/2 inbred mice (13).

Female hybrid mice, B6C3F₁ (C57BL/6 female × C3H male) and CD2F₁ (BALB/c female × DBA/2 male) were purchased from the Animal Genetics and Production Branch, NCI. The mice were 5 to 6 weeks old and weighed 17 to 21 g. The mice were maintained in

conformity with the standard guidelines (14). They were housed in plastic cages (four to five mice per cage) with hardwood chip bedding and provided with dry pelleted mouse feed and water *ad libitum*. All mice were quarantined for 1 week prior to randomization. The mice were regularly inspected for gross signs of pathogenic conditions and injury.

The allogeneic mice were randomized four to a cage after the quarantine period. The cages were further randomized prior to assigning to a treatment group. Each treatment group consisted of two cages or a total of six to eight animals. Each animal in a cage was ear-marked for individual identification. The syngeneic mice were randomized immediately before tumor challenge.

L1210 ascitic fluid was harvested aseptically from healthy donor mice and placed in a sterile glass container on ice. A cell count was made using a hemocytometer, and the stock fluid was diluted in Hank's balanced salt solution to the desired cell concentration. The number of cells implanted intraperitoneally (3×10^6 , 1×10^5 , 3×10^3 , or 1×10^2 per mouse) was contained in 0.2 mL cell suspension.

Group body weights were taken once a week during the experimental period. Survival, general health conditions, and other clinical observations were recorded daily. Necropsies were performed to record the presence of leukemic ascites, splenomegaly, and hepatomegaly. Smears were made from retro-orbital sinus blood from each animal on the last day of dosing and on the day of death (or at 43 days post-tumor implantation for the allogeneic mice.) These smears were stained with Giemsa and differential cell counts were made.

The chemicals were prepared in appropriate concentrations and were administered to mice perorally in a volume of 0.01 mL/g body weight. The allogeneic B6C3F₁ mice were treated daily from day -12 to day -8 and from day -5 to day -1. Day 0 was the tumor implantation day for both strains of mice. Cyclophosphamide at a dose of 180 mg/kg was injected intraperitoneally on day -1. The syngeneic CD2F₁ mice were treated daily on days 1 to 5 and days 8 to 12. There was no cyclophosphamide-treated group in the syngeneic mice. The negative control group for each strain was

Table 1. Effect of EGEE or EGME on L1210 leukemia in syngeneic mice.

Treatment ^a	Dose, mg/kg/adm	Avg. body weight change, g	Death range, days	MST, days ^b
EGEE	2400	1.7	8	8
	1200	1.7	8-9	8
	600	5.7	8	8
EGME	1200	2.3	9-10	9.5
	600	1.9	9-10	9
	300	1.5	9	9
Water	—	4.7	8-9	8

^aChemicals and water were administered on days 1-5 and 8-12 (or until the animals died) after a tumor challenge of 1×10^5 L1210 leukemia cells. Eight animals per group.

^bMedian survival time.

Table 2. Effects of EGME on L1210 leukemia in allogeneic mice.

Treatment ^a	Dose, mg/kg/adm	L1210 tumor inoculum	Avg. body weight change, g	MST, days	Survivors/total
EGME	1200	3×10^6	6.6	> 43	7/7
	600	3×10^6	7.1	> 43	7/8
	300	3×10^6	6.8	> 43	7/8
	1200	1×10^5	6.3	> 43	5/6
	600	1×10^5	5.9	> 43	6/8
	300	1×10^5	7.1	> 43	7/7
	1200	3×10^3	6.9	> 43	7/7
	600	3×10^3	6.5	> 43	7/7
	300	3×10^3	6.0	> 43	7/7
	1200	1×10^2	5.9	> 43	7/7
	600	1×10^2	6.4	> 43	6/6
	300	1×10^2	6.0	> 43	7/7
Cyclophosphamide	180	3×10^6	—	7	0/8
	180	1×10^5	6.3	> 43	8/8
	180	3×10^3	6.3	> 43	8/8
	180	1×10^2	6.2	> 43	8/8
Water	—	3×10^6	—	9	0/7
	—	1×10^5	5.2	> 43	8/8
	—	3×10^3	5.0	> 43	6/6
	—	1×10^2	6.2	> 43	6/6

^aEGME and water were administered by gavage on days -12 through -8 and -5 through -1 before challenge with L1210 cells. Cyclophosphamide was administered 1 day prior to tumor challenge.

Table 3. Effects of EGEE on L1210 leukemia in allogeneic mice.

Treatment ^a	Dose, mg/kg/adm	L1210 tumor inoculum	Avg. body weight change, g	MST, days	Survivors/total
EGEE	2400	3×10^6	6.7	> 43	5/6
	1200	3×10^6	6.7	> 43	5/6
	600	3×10^6	7.0	> 43	7/8
	2400	1×10^5	12.5	> 43	5/6
	1200	1×10^5	8.8	> 43	4/5
	600	1×10^5	6.1	> 43	6/7
	2400	3×10^3	7.3	> 43	7/7
	1200	3×10^3	7.3	> 43	7/7
	600	3×10^3	6.3	> 43	8/8
	1200	1×10^2	6.0	> 43	7/7
	600	1×10^2	7.0	> 43	7/7
Cyclophosphamide	180	3×10^6	—	7	0/8
	180	1×10^5	6.3	> 43	8/8
	180	3×10^3	6.2	> 43	8/8
	180	1×10^2	6.2	> 43	8/8
Water	—	3×10^6	—	9	0/7
	—	1×10^5	5.2	> 43	8/8
	—	3×10^3	5.0	> 43	6/6
	—	1×10^2	6.2	> 43	6/6

^aEGEE and water were administered by gavage on days -12 through -8 and -5 through -1 before challenge with L1210 cells. Cyclophosphamide was administered 1 day prior to tumor challenge.

given distilled water on the days corresponding to the chemical treatment days.

Results

The survival and weight results for the experiment are presented in Table 1 for the syngeneic mice. The challenge with 1×10^5 L1210 cells in water-treated mice produced deaths, with a median survival time (MST) of

8.0 days. This is the acceptable range (8–11 days) for the L1210 tumor at this challenge level. There was no pronounced effect of treatment with either drug at any dose level on the prolongation of life. The groups treated with the EGME did survive slightly longer than the EGEE-treated group and the water-treated controls. These differences in survival were small and are not considered biologically significant in this tumor model by NCI standards. However, pairwise compari-

sons (two-sample *t*-test) showed that the differences are statistically significant ($p < 0.0001$). No toxicity was seen as assessed by body weight change with either chemical at any dose.

Necropsy of syngeneic mice showed hemorrhagic ascites and splenomegaly. Liver, lungs, and kidneys were normal in gross appearance for most animals. Groups which received the top two doses of the EGME compound, each had one mouse with no visible ascites and two each with a small quantity of ascites.

With the allogeneic mice, all animals died at the 3×10^6 challenge level in the cyclophosphamide-treated group and the water-treated controls (Tables 2 and 3). These groups differed significantly ($p < 0.0001$) from the 3×10^6 challenge groups treated with EGME (Table 2) or with the EGEE (Table 3) where no more than one animal died in each group. No mice challenged with $1 \times$

10^5 , 3×10^3 , or 1×10^2 L1210 cells died in the water treatment groups. Likewise, no mice died at those challenge levels in the cyclophosphamide-pretreated mice. However, the mice which were given cyclophosphamide and challenged with 1×10^5 cells, developed ascites as noted by abdominal distention. This developed at day 7 and was noted until approximately day 14, when all of the ascites was resorbed and the mice continued to live until termination on day 43. Necropsy of these animals showed no signs of tumor.

In allogeneic mice treated with EGME compound, one animal each died at the two lower treatment doses with the 3×10^6 challenge level while one animal died at the top treatment dose and two died at the second dose at 1×10^5 challenge. One animal died in the group which received the 600 mg/kg dose of EGME compound and was challenged with 3×10^3 cells. All mice that died in these groups had either ascites, splenomegaly, and/or hepatomegaly. With the EGEE compound, only one death in each group at the 3×10^6 and 1×10^5 level occurred, while no deaths occurred at any dose level with the 3×10^3 and 1×10^2 challenge level (Table 3). All surviving allogeneic mice were sacrificed on day 43 for necropsy and blood smears. No mice showed any ascites and only 4 (6%) showed splenomegaly. Of the 67 mice treated with the EGEE that survived, 5 (7%) had enlarged gallbladders, while of the 79 mice treated with the EGME compound that survived, 46 (58%) had enlarged gallbladders. No surviving animals that received cyclophosphamide or water had enlarged gallbladders.

The data from the blood counts is seen in Tables 4 to 6. In general, the morphology of the "leukemic cells" appeared as atypical, vacuolized mononuclear cells with characteristic red granules. Syngeneic mice died with evidence of monocytosis which is presumed to be indicative of monocytic leukemia (Table 4). Most of these treated animals were sacrificed and bled on day 43 after tumor challenge. A few mice treated with EGME

Table 4. Incidence of monocytic leukemia by differential cell count in syngeneic mice treated with EGME or EGEE.

Treatment group ^a	Dose, mg/kg	No. ^b	Differential count ^c	
			HM	L
EGME	1200	7	0	7
	600	7	0	6
	300	6	0	6
EGEE	2400	8	0	8
	1200	7	1	5
	600	6	0	6
Water	—	7	0	6

^aAll mice treated with 1×10^5 cells tumor challenge. Where there were less than 8 initial mice per group, the animals died before the blood sample could be obtained.

^bNo. = initial number of mice.

^cCriteria: normal = 0-8% monocytes; high = 9-14% monocytes; monocytosis indicative of monocytic leukemia $\geq 15\%$ monocytes. HM = high monocyte count. L = monocytosis presumed indicative of monocytic leukemia.

Table 5. Incidence of monocytic leukemia by differential cell count in allogeneic mice treated with EGME.^a

Treatment group	Pretumor challenge			Tumor challenge ^a															
				3×10^6 cells/mouse				1×10^5 cells/mouse				3×10^3 cells/mouse				1×10^2 cells/mouse			
	No. ^b	HM ^b	L ^b	Ns ^b	HM ^b	L ^b	D ^b	Ns	HM	L	D	Ns	HM	L	D	Ns	HM	L	D
EGME																			
1200 mg/kg	26	0	1	7	0	0*	—	5	0	0	1	7	0	0	—	6	0	0	—
600 mg/kg	30	0	2	7	0	0*	1	6	1	0	2	6	0	0	1	7	0	0	—
300 mg/kg	29	0	0	7	0	0*	1	7	0	0	—	7	0	0	—	7	1	0	—
Water	27	0	0	6	1	5†	1	8	0	0	—	6	0	0	—	6	0	1	—
CYC ^d	32	0	0	—	—	—	8	8 ^c	0	0	—	8	0	0	—	8	0	0	—

^aCriteria: Normal = 0-8 % monocytes; high = 9-14% monocytes; monocytosis indicative of monocytic leukemia $\geq 15\%$ monocytes.

^bNo. = initial number of mice = Ns + D; Ns = number of mice sampled; HM = High monocyte count; L = monocytosis presumed indicative of monocytic leukemia; D = died without blood sample.

^cAs noted in the text, this group developed ascites at day 7 which was noted until approximately day 14 when all of the ascites was resorbed and the mice survived until sacrifice on day 43.

^dCYC = cyclophosphamide (180 mg/kg).

*Significantly different from water control ($p < 0.005$).

†Significantly different from pretumor challenge ($p < 0.00001$).

Table 6. Incidence of monocytic leukemia by differential cell count in allogeneic mice treated with EGEE.

Treatment group	Pretumor challenge			Tumor challenge ^a															
				3×10^6 cells/mouse				1×10^5 cells/mouse				3×10^3 cells/mouse				1×10^2 cells/mouse			
	No. ^b	HM ^b	L ^b	Ns ^b	HM ^b	L ^b	D ^b	Ns	HM	L	D	Ns	HM	L	D	Ns	HM	L	D
EGEE																			
2400 mg/kg	18	1	2	5	2	0*	1	5	1	0	1	6	1	1	—		Not done		
1200 mg/kg	25	0	1	5	0	0*	1	4	0	1	1	7	0	0	—	7	2**	0	—
600 mg/kg	30	2	1	7	0	0*	1	6	0	0	1	8	1	0	—	7	0	0	—
Water	27	0	0	6	1	5†	1	8	0	0	—	6	0	0	—	6	0	1	—
CYC ^c	32	0	0	—	—	—	8	8 ^d	0	0	—	8	0	0	—	8	0	0	—

^aCriteria: normal = 0–8% monocytes; high = 9–14% monocytes; monocytosis presumed indicative of monocytic leukemia \geq 15% monocytes.

^bSee Table 5.

^cCYC = cyclophosphamide (180 mg/kg).

^dAs noted in the text, this group developed ascites at day 7 which was noted until approximately day 14 when all of the ascites was resorbed and the mice survived until sacrifice on day 43.

*Significantly different from water control ($p < 0.02$).

†Significantly different from water control ($p < 0.005$).

‡Significantly different from pretumor challenge ($p < 0.00001$).

**Significantly different from pretumor challenge ($p < 0.05$).

or EGEE (Tables 5 and 6) but not challenged with tumors showed evidence of monocytosis. Since no gross pathological observations were made at the time of the first bleeding, one can only speculate on the cause. It is reasonable to assume that penetration of the esophagus or trachea may have occurred due to dosing error resulting in an infection. However, of the four mice that died in the EGEE group, two who had monocytosis at the first bleeding died with evidence of leukemia (one in the 1200 and one in the 2400 mg/kg group). This also occurred in one mouse treated with 600 mg/kg EGME. The remaining animals with evidence of monocytosis at the first bleeding had a normal blood profile at sacrifice.

Discussion

The results shown for the syngeneic mice demonstrate that there is probably no direct antitumor activity of the compounds against the L1210 tumor at the doses or schedule which were evaluated. At the same time there was no toxicity as assessed by weight loss or early death. This could indicate that higher dosages might be tolerated and have some direct cytotoxic effect on the tumor.

The results for the allogeneic mice are somewhat more complex. It is apparent that the 3×10^6 tumor challenge level was able to overcome the allogeneic rejection phenomenon in that all water-treated controls died, as did the cyclophosphamide-pretreated mice. The mice treated with cyclophosphamide and challenged with 1×10^5 cells developed ascites, but were still able to overcome tumor by rejection. The fact that most of the allogeneic mice pretreated with either test compound and challenged with 3×10^6 L1210 cells did not die would seem to indicate that the compounds have some type of prophylactic action or may in some way actually stimulate the immune system, since the comparable control mice died with this challenge.

The results are interesting in that they appear to

support the observations of overall decreases in background gross pathology of the spleen, pituitary, testes or mammary gland in aging Fischer 344 rats administered EGEE by oral gavage (15). The current study suggests that EGEE and EGME may exert an antitumor effect through increased immunological competence or immunomodulation.

To more accurately determine what immunologic effects these compounds have in an allogeneic tumor model, in comparison with the standard immunosuppressive compound cyclophosphamide, an experiment with the following challenge levels is suggested: 5×10^6 , 1×10^6 , 5×10^5 , 1×10^5 . This should produce groups where the cyclophosphamide treated groups will die, but the water-treated controls will not (1×10^6 and 5×10^5). Further, higher doses of the compounds that would produce pronounced toxic effects (e.g., death, weight loss, bone marrow toxicity), as well as additional assays to assess immune competence, should be included.

Since these glycol ethers exhibit relatively low toxicity in organ systems other than the reproductive tract, are readily absorbed by various routes of administration, and appear to decrease background gross pathology, there may be enough evidence to warrant testing of these chemicals as potential therapeutic agents.

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